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Original Article

Nectandra falcifolia: potential phytopharmaceutical for skin damage protection designed by statistical approach and characterized by photoacoustic spectroscopy

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ABSTRACT

Phytopharmaceutical products are being used in the treatment and prevention of health problems. Nowadays, the development and evaluation of novel pharmaceutical products is expensive and time consuming. A statistical approach is a good tool for optimal development processes. *Nectandra falcifolia* (Nees) J.A. Castigl. ex Mart. Crov. & Piccinini, Lauraceae, a Brazilian species, is reported as anti-inflammatory, anti-leishmanial and anti-microbial. However, there is little known about its chemical composition. For other species of *Nectandra* genus, the presence of antioxidant compounds is reported. In order to optimize the process of obtaining extract with high antioxidant activity, different extraction conditions were tested following a statistical approach. Two sequential experimental designs were used – first, a factorial 2^3 design, followed by central composite 2^2. The extracts manufactured by these experimental statistical matrixes had their antioxidant activity and phenolic contents quantified and the response surface plots were fitted in quadratic models and they predicted the best extraction condition for the best antioxidant activity. This standardized extract and its antioxidant activity were better evaluated by two complementary tests (ABTS and Burst respiratory). A topical formulation containing 1% (w/w) of standardized extract was prepared and used for an *in vivo* skin permeation study using a two-dose application. The photoacoustic spectroscopy was used to analyze the samples from the permeation study and the composition profile of standardized extract. In rat skin samples, the data demonstrated that for the higher dose of topical formulation (5 g/cm^2), the standardized extract could cross skin and be seen in epidermis and dermis. This was not the case for the lower dose (2 g/cm^2) which was only present in the epidermis. This information suggests that this novel standardized extract of *N. falcifolia* could be explored for skin damage prevention or treatment for diseases developed by oxidative damage.

Introduction

Currently, many studies are conducted to obtain more information about antioxidative activity of plant materials and their derivatives. These resources have been frequently used in topical formulations to prevent cell oxidative damage (Lonni et al., 2012).

In a bioprospecting approach to species popularly known as "canelinha" (*Nectandra falcifolia* (Nees) J.A. Castigl. ex Mart. Crov. & Piccinini, Lauraceae), studies have shown that extracts from this species exhibited anti-inflammatory, anti-leishmanial and antibacterial activities (Oliveira de Melo et al., 2006; Truiti et al., 2005, 2006). About phytochemical profile, no information about *N. falcifolia* was found, but other species of the same genus (*Nectandra*

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grandiflorus) have shown high content of flavonoids (Ribeiro et al., 2002). Both species are distributed in many tropical and subtropical areas of the planet, mainly in Southwest Asia and Brazil (Oliveira de Melo et al., 2006).

Flavonoid and other phenolic compounds are known for diverse biological activities, including antioxidant activity by scavenging of free radicals and also by the inhibition of the lipid peroxidation (Hanasaki et al., 1994). These biological activities increase scientific and economic attention for herbal drugs containing phenolic to be used for topical formulation in prevention or recovery of skin damage.

In the meantime, there are several ways to obtain herbal extracts. Distinct extractive systems can generate different products in terms of phenolic contents, even when made from the same herbal species (Audi et al., 2001; Lapornik et al., 2005).

Because these uncountable numbers of variations in productive and analytic processes of a plant extract, the first step for the development of new herbal extract should be to optimize and standardize the manufacture procedures. For this purpose, the response surface methodology (RSM) has been a very useful tool in improving the process. It is widely used to assess the relationship between chemical contents and biological activities. RSM is also used to understand interactions between variables to obtain a better activity (Onsekizoglu et al., 2010).

Presently, characterization of extract composition has been reported using different analytical techniques, from traditional spectroscopy (Berbicz et al., 2011) to modern methods like UPLC–MS/MS (Oszmiański et al., 2014). New methods are being applied in scientific scenarios every day and the choice for the better technique is case-dependent.

In this way, photoacoustic spectroscopy (PAS) was described as a technique that can be used to biological/pharmaceutical analysis for different samples matrix. The PAS can be very efficient to evaluate the permeation and distribution of substances through the skin *in vitro*, *ex vivo* and *in vivo* (Truite et al., 2007). PAS is based on optical absorption spectra determined by photoacoustic signal generated by the interaction of matter with a radiation of known wavelength (Berbicz et al., 2011; Truite et al., 2007). This can be used to analyze the depth of skin permeation profile of formulations, including herbal extract formulations (Truite et al., 2007).

Materials and methods

Materials

All reagents and solvents used were analytical grade, including gallic acid (Sigma®), ethyl alcohol P.A. (Synth®), methanol P.A. (F Maia®), butylatedhydroxy toluene (BHT) (Aldrich®), anhydrous sodium carbonate P.A. (Neon®), aluminum chloride P.A. (Synth), 2,2-diphenyl-1-picryl-hydrazyl (Aldrich®), Folin Ciocalteau (Dinâmica®) and quercetin (Sigma®).

Raw material

Leaves of the *Nectandra falcifolia* (Nees) J.A. Castigl. ex Mart. Crov. & Piccinini, Lauraceae, were collected in Taquaraçu, MS, Brazil. The material was herborized and deposited in Nupélia herbarium/Universidade Estadual de Maringá, PR, Brazil with wide voucher specimen number HNUP n° 1421. The material was dried at 40 °C in a hot-air oven, and ground by blade mill (d = 1.6 mm). The herbal drug characterization (water and ash containing and granulometric analysis) was made (WHO, 2011). The method used for the extracts production was maceration.

Experimental statistical design

Two sequential experimental matrices were used. The first one was a factorial design 2^3 (Table 1). In order to produce extracts, the variables were: extraction time (ET) (h), ethanol concentration (EC) (%) and plant proportions (PP) (w/v) which were applied in two levels and a central point (n = 4). The total phenolic contents and antioxidant activity were quantified for each extract produced. After statistical analysis of the first data set, the ethanol concentration was fixed in 100% and the other variables were optimized following the second experimental matrix, a central composite design 2^2 (Table 1).

For both designs, all extracts were produced at random (n = 3). The alcohol was evaporated under reduced pressure at 40 °C, lyophilized and stored in amber bottles protected from light, heat and moisture, while waiting for analysis. The software Statistica 7.0® (StatSoft, USA) was used to compare the results.

Radical scavenging activity DPPH assay

The antioxidant activity of the lyophilized extracts was quantified by the photocolorimetric method of the free radical DPPH (Prior et al., 2005). This method was adapted and validated. To run the analysis, stock solution were produced by dissolving the extracts in methanol (5 ml). Analyses were performed in triplicate.

From stock solutions, six concentrations were prepared (20, 24, 28, 32, 36, 40 μg/ml). Two milliliters of the reacting solution of DPPH (0.06 mg/ml) were added to 1.0 ml of each of these concentrations. The time of reaction was 30 min. The UV spectrometer was calibrated using a blank solution (3 ml of methanol P.A.) and the negative solution (no reaction) was composed of 1 ml of methanol, added to 2 ml of DPPH reacting solution. The wavelength used was 516 nm.

The percentage of inhibition was calculated according to Eq. (1),

$$\text{IC}\ (\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100 \quad (1)$$

where A_0 is the absorbance of negative solution and A_1 is the absorbance of samples reactions.

The values of the IC_{50} were estimated by linear regression from the IC (%) curve versus concentrations.

Determination of the total phenolic content

The total phenolic content was quantified according to the method of Sousa et al. (2007) which was adapted and validated. Twenty milligrams of each dried extract were dissolved in methanol PA and the experiment was performed. The blank solution (methanol and the other reagents, except the extract) was also analyzed and all samples were read at 760 nm at room temperature.

The calibration curve was built for gallic acid (a standard phenolic compound) using the same reaction conditions. The total phenolic content was determined by interpolating the absorbance of the samples against a calibration curve for gallic acid. The total phenolic content acid was expressed in equivalent milligrams per gram of dry extract (mgGAE/g of extract). All assays were performed in triplicate.

Determination of the total flavonoid content

The total flavonoid content in the dried extracts was determined according to the Brazilian pharmacopoeia (monograph of the *Calendula officinalis* L., Asteraceae) (Anvisa, 2010), again adapted and validated.

For this study, the calibration curve was built up using quercetin. Five milligrams of dried extract were used for the reaction. The

Table 1
Factorial Design Matrix 2^3 and Central Composite Design Matrix 2^2 for the optimization of the total phenolic contents and antioxidant activity of extracts of *Nectandra facifolia* leaves.

Factorial Design Matrix 2^3						Central Composite Design Matrix 2^2			
X1*	X2*	X3*	X1PP (w/v)	X2ET (h)	X3EC (%)	X1*	X2*	X1ET (h)	X2PP (w/v)
1.00	1.00	1.00	1:20	72	100	−1.00	−1.00	24	1:10
1.00	1.00	−1.00	1:20	72	50	−1.00	1.00	24	1:20
1.00	−1.00	1.00	1:20	24	100	1.00	−1.00	72	1:10
1.00	−1.00	−1.00	1:20	24	50	1.00	1.00	72	1:20
−1.00	1.00	1.00	1:10	72	100	−1.4142	0.00	14	1:15
−1.00	1.00	−1.00	1:10	72	50	1.4142	0.00	82	1:15
−1.00	−1.00	1.00	1:10	24	100	0.00	−1.4142	48	1:08
−1.00	−1.00	−1.00	1:10	24	50	0.00	1.4142	48	1:22
0.00	0.00	0.00	1:15	48	75	0.00	0.00	48	1:15
0.00	0.00	0.00	1:15	48	75	0.00	0.00	48	1:15
0.00	0.00	0.00	1:15	48	75	0.00	0.00	48	1:15
0.00	0.00	0.00	1:15	48	75	0.00	0.00	48	1:15

X1*, X2*, X3*: coded variable; X1, X2, X3: decoded variable.

total flavonoid contents was determined applying the samples absorbance to the quercetin curve equation and expressed as milligrams of quercetin equivalent by gram of dried extract (mg EQ/g of extract). Assays were performed in triplicate.

Complementary antioxidative activity assays for standardized extract.

Free radicals scavenger activity (ABTS) assays

The antioxidative capacity of the standardized extract was measured in comparison to the free radical ABTS+• (Sánchez-González et al., 2005). The ABTS+• cation was produced by the reaction of 7 mM of the stock solution ABTS with 2.45 mM of potassium persulfate. The ion ABTS+• solution was diluted with the phosphate buffer (pH 7.4) and read for absorbance (0.7–730 nm). The mass extracts (20, 30 and 40 μg) were solubilized in dimethylsulfoxide (DMSO). The reaction was made by adding 10 μl of the samples to 4 ml of the ABTS+• solution diluted. The samples were read at 730 nm after 6 min of reaction. The antioxidant capacity was calculated by the percentage of inhibition of the radical ABTS (IA %), according to Eq. (2):

$$\text{IA}\ \ (\%) = 100 - \left(\frac{A_1}{A_0}\right) \times 100 \qquad (2)$$

where A_1 is the value of absorbance of the sample and A_0 is the value of the absorbance of the control solution in absence of the sample (Sánchez-González et al., 2005).

Respiratory Burst methodology

The respiratory burst was performed using human neutrophils to evaluate the kinetic production of oxygen reactive species after stimulation by phorbol-myristate (PMA). Chemioluminescence was monitored in a microplate reader Victor X3 (PerkinElmer®, EUA) (Wymann et al., 1987). The results were expressed in counts per minute (CPM). For samples of standardized *N. falcifolia* extract, the concentrations tested were: 20, 30 and 40 μg/ml. Results were shown according to the values of the peaks from the kinetic curves and presented in the form of quartiles (1st and 3rd). Tests were performed in 14 repetitions.

Topical formulation containing the standardized extract (TPSE)

A non-ionic emulsion base formulation was developed with the following composition: distilled water, propyleneglycol, ceteareth-20; glyceryl stearate; cetearyl alcohol; decyloleate; caprylic/capric triglyceride; methylparaben; propylparaben. The emulsion was produced by a conventional technique in a laboratory setting. Subsequently, the standardized *N. falcifolia* extract (SNFE) was incorporated in 1% of the base formulation.

Photoacoustic spectroscopy (PAS)

The SNFE, base formulation and TPSE were analyzed by photoacoustic spectroscopy. This was done to verify the spectral response of the components in ultraviolet and visible spectra in each sample. The measurement was performed using an experimental setup (Baesso et al., 1994; Berbicz et al., 2011; Oliveira De Melo et al., 2011). The monochromatic light (xenon arc lamp – Oriel, Model 68820) with a power of 10^3 watts (W) and an emission in the range 180 to 4×10^3 nm, and a monochromator (Oriel, Model 77250). The frequency of the light modulation was controlled by a mechanical modulator (Stanford Research Systems, Model SR540). The microphone (Brüel & Kjaer, BK Model 2669) was attached to the photoacoustic cell and connected to a power source and preamplifier. The signal from the microphone was transferred to the Lock-in (tuned amplifier) (EG & G Instruments Model 5110). The Lock-in provides the intensity and phase of the photoacoustic signal which is transferred to a microcomputer via GPIB interface. The photoacoustic spectra were obtained by modulation frequencies of 25 Hz, power of 700 W and were recorded between 200 and 800 nm.

Evaluation of percutaneous permeation profile of topical formulations by photoacoustic spectroscopy (PAS)

The penetration profiles of topical formulations (base and TPSE) were evaluated by PAS (Baesso et al., 1994; Oliveira De Melo et al., 2011). The experimental procedure involving the use of animals was approved by the Ethics Committee on Animal Use in Experimentation, Universidade Estadual de Maringá (n° 100/2012).

Male Wistar rats, weighing 150–200 g, were divided into three groups and exposed to: (G1) base formulation (dose applied was 2 mg/cm^2 ($n = 3$)); (G2) base formulation (dose 5 mg/cm^2 ($n = 4$)); (G3 and G4) TPSE in two applications (2 mg/cm^2 and 5 mg/cm^2), respectively.

After anesthetized with Tiopental (40 mg/kg), the hair next to the cervical region was removed by plucking. Base and TPSE formulations were applied to the clean skin surface. About 30–45 min after application, the rats were euthanized by anesthesia (Tiopental 120 mg/kg) and their skins were removed for analysis. The skins were dissected in thicknesses between 1.19 and 1.74 mm comprising the epidermis and dermis, and percutaneous permeation measurements were performed 20 min after the death of the animals.

Table 2
Levels of phenolic total content (TP) (mgGAE/g extract) and IC_{50} of the antioxidant activity (AA) from the *Nectandra falcifolia* extracts obtained by the factorial 2^3 and Central Composite 2^2 designs.

Factorial Design 2^3					Central Composite Design 2^2			
X1 ET	X2 EC	X3 PP	TP Y1	AA (DPPH) Y2	X1 ET	X3 PP	TP Y1	AA (DPPH) Y2
24	50	01:10	141.9 ± 18.4	27.7 ± 1.2	24	1:10	765.4 ± 42.6	24.7 ± 1.1
24	50	01:20	231.8 ± 28.5	19.4 ± 0.7	24	1:20	529.8 ± 2.5	27.1 ± 0.3
24	100	01:10	765.4 ± 42.7	24.7 ± 1.1	72	1:10	864.8 ± 1.7	19.8 ± 0.6
24	100	01:20	529.8 ± 2.5	27.1 ± 0.3	72	1:20	567.2 ± 46.9	23.2 ± 0.5
72	50	01:10	278.3 ± 26.6	21.5 ± 1.6	14	1:15	136.9 ± 1.17	32.9 ± 0.4
72	50	01:20	385.5 ± 33.3	26.8 ± 0.7	82	1:15	757.1 ± 10.2	21.4 ± 0.1
72	100	01:10	864.8 ± 1.72	19.8 ± 0.6	48	1:08	903.2 ± 24.7	22.8 ± 0.4
72	100	01:20	567.2 ± 46.9	23.2 ± 0.5	48	1:22	520.5 ± 49.1	30.9 ± 0.5
48	75	01:15	74.5 ± 19.5	22.3 ± 0.1	48	1:15	655.5 ± 13.2	23.2 ± 0.8
48	75	01:15	104.5 ± 25.0	22.4 ± 0.2	48	1:15	648.3 ± 1.1	24.2 ± 0.4
48	75	01:15	94.95 ± 1.0	22.9 ± 0.4	48	1:15	641.2 ± 26.7	22.8 ± 0.7
48	75	01:15	72.25 ± 1.8	23.1 ± 0.1	48	1:15	647.7 ± 1.8	23.9 ± 0.7

Data are mean ± SD, $n = 3$.
X1: ET – extraction time; X2: EC – ethanolic concentration; X3: PP – plant proportions; X1, X2, X3: decoded variable; Y1, Y2: average ± sd; TP: total phenolic (mgGAE/g ext); AA: DPPH-IC50 (μg/ml).

All absorption spectra were obtained using a modulation frequency of 25 Hz in the range of 200–800 nm region of the ultraviolet spectrum (UV) and visible (VIS).

Results and discussion

The first matrix projected was the factorial 2^3 experimental design. Results for total phenolic contents and antioxidant activity by DPPH method of the extracts are presented in Table 2. Total phenolic contents are expressed in terms of milligrams of gallic acid per gram of dried extract (mgGAE/g extract) and antioxidant activity is expressed in a concentration of fifty percent of inhibition (IC_{50}). ANOVA was performed to the models generated by the factorial 2^3 for both dependent variables and the results are presented in Table 3.

The performance of the extracts designed by this matrix was fitted in a linear model for dependent variables. For total phenolic content, the adjusted model presented the overall mean 470.63 ± 5.31 mgGAE/g extract. The main effects were: ET −84.04 ± 10.62 mgGAE/g extract, EC 422.41 ± 10.62 mgGAE/g and PP 106.74 ± 10.62 mgGAE/g. Significant interaction effects between ET × EC was −182.55 ± 10.62 mgGAE/g extract and for EC × PP was −38.31 ± 10.62 mgGAE/g extract. The R^2 was 0.99 and the R^2 Adjusted was 0.99.

These results show that ethanol concentration (EC) was the most important variable for better extractive efficiency of total phenolic. The higher concentration of ethanol presented better phenolic levels in the extract. On the other hand, the interaction between ET × EC showed negative effect, as did the interaction ET and PP. These negative signs indicate a negative contribution decreasing extractive efficiency when the variables EC and PP are increased.

Regarding antioxidant activity, the overall mean of IC_{50} was 23.86 ± 0.16 μg/ml. The main effects were for ET was 0.71 ± 0.32, EC was −0.13 ± 0.32 and for PP −1.93 ± 0.32 μg/ml. Significant interaction effects were also found. They were 2.17 ± 0.32 μg/ml (ET × EC), 3.67 ± 0.32 μg/ml (ET × PP), −2.48 ± 0.32 μg/ml (EC × PP) and −3.14 ± 0.32 μg/ml (ET × EC × PP). Thus, the most important factor for the best result for decreasing IC_{50} was PP. However, the best response for decreasing IC_{50} values was the interaction between PP × EC and ET × EC × PP. The improvement in antioxidant capacity is related to the increasing of EC, ET and PP levels because they presented negative interaction effects. The R^2 was 0.94 and the R^2 Adjusted was 0.92.

Results show that the optimal extracts were obtained using EC 100%. Additionally, in statistical analysis the curvature effect was significant and indicated that the next step needed to be the rotation on the matrix plane to characterize the existence of quadratic effect. Whereas the rotation axis led to the establishment of axial points (−1.4142 and +1.4142) to this model (Table 2), the upper axial point of execution was not possible for EC because it was already tested in a maximum concentration (100%) level +1.

Since this point, the variable EC was fixed in 100% and was designed the second experimental matrix, a central composite 2^2. The new matrix is presented in Table 1 and the results for these extracts are presented in Table 2. ANOVA was performed to the models generated by central composite 2^2 for both dependent variables and the results are presented in Table 4. The models adjusted for the second data set are presented in Table 5.

Table 3
ANOVA results to the model generated by the factorial 2^3 for total phenolic content and for total antioxidant activity.

Factors	Total phenolic content					Antioxidant activity				
	SS	df	MS	*F*	*p*	SS	df	MS	*F*	*p*
Curve	1,179,925	1	1,179,925	1741.9	0.00	10.32	1	10.32	16.4	0.00
ET	42,384	1	42,384	62.5	0.00	3.10	1	3.10	4.9	0.03
EC	1,070,591	1	1,070,591	1580.5	0.00	0.10	1	0.10	0.2	0.67
PP	68,369	1	68,369	100.9	0.00	22.57	1	22.57	35.8	0.00
ET×EC	199,966	1	199,966	295.2	0.00	28.39	1	28.39	45.0	0.00
ET×PP	746	1	746	1.10	0.30	81.12	1	81.12	128.5	0.00
EC×PP	8807	1	8807	13.0	0.00	36.97	1	36.97	58.6	0.00
ET×EC×PP	2366	1	2366	3.49	0.07	59.31	1	59.31	94.0	0.00
Pure error	18,289	27	677			17.04	27	0.63		
Total SS	2,591,443	35				258.9	35			

SS: sum square; df: degree of freedom; MS: mean square; ET: extraction time; PP: plant proportion.

Table 4
ANOVA results to the model generated by central composite 2^2 for total phenolic and for total antioxidant activity.

Factors	Total phenolic content					Antioxidant activity				
	SS	df	MS	*F*	*p*	SS	df	MS	*F*	*p*
ET (L)	385,439	1	385,439	633.42	0.00	235.40	1	235.4	469.42	0.00
ET (Q)	108,006	1	108,006	177.49	0.00	18.45	1	7.37	18.45	0.00
PP (L)	432,859	1	432,859	711.35	0.01	111.61	1	111.6	222.56	0.00
PP (Q)	63,384	1	63,384	104.16	0.00	14.02	1	14.0	27.96	0.00
ET(L) × PP(L)	2884	1	2884	4.74	0.10	0.85	1	0.8	1.69	0.20
Lack of fit	268,302	3	89,434	146.97	0.03	97.84	3	32.6	65.03	0.00
Pure error	16,429	27	608.5			13.53	27	0.5		
Total SS	1,318,920	35				486.38	35			

ET (L) × PP (L): interaction ET (L) and PP (L); SS: sum square; df: degree of freedom; MS: mean square; ET: extraction time; PP: plant proportion; L: linear term; Q: quadratic term.

In the second set of experiments, data of total phenolic content of the overall mean value was 648.19 ± 7.12 mgGAE/g extract, and significant main effects were 253.45 ± 10.07 mgGAE/g extract for ET linear (L), −150.00 ± 11.25 mgGAE/g extract for ET quadratic (Q), −268.59 ± 10.07 mgGAE/g extract for the PP(L) and 114.91 ± 11.25 mgGAE/g extract for PP(Q). None meaningful interaction effect was observed. The R^2 was 0.79 and the R^2 Adjusted was 0.75.

For the antioxidant values in the overall average IC_{50} was 23.58 ± 0.20 μg/ml, and the significant variable effects were −6.26 ± 0.28 μg/ml for ET (L), 1.96 ± 0.32 μg/ml for ET(Q), 4.31 ± 0.28 μg/ml for PP(L), 1.70 ± 0.32 μg/ml for PP(Q). As for total phenolic content, none significant interaction effect was observed. The R^2 was 0.78 and the R^2 Adjusted was 0.73.

Response surfaces graphs from central composite 2^2 for the total phenolic content (A) and antioxidant activity (IC_{50}) (B) of the *N. falcifolia* extracts are shown in Fig. 1.

The combined optimal value for the total phenolic content and antioxidative activity of the *N. falcifolia* extracts was predicted for extract prepared with ET 100%, ET 72 h and PP 1:10 (w/w).

The extract produced under these conditions was called standardized *N. falcifolia* extract (SNFE). It was produced, dried and stored under the same conditions previously described. This extract had its antioxidant activity evaluated by two more methods (ABTS and respiratory burst) in order to explore better this property.

The ABTS method has a different antioxidant mechanism in comparison to DPPH. Percentage of inhibition (%) for the SNFE was presented to compare results. The masses of extract used for both methods were 20, 30 and 40 μg. For 40 μg the DPPH inhibition (%) was 88.13 and the ABTS inhibition (%) was 93.50, for 30 μg was the DPPH 67.92 and the ABTS 93.10 and for 20 μg the DPPH 47.71 and the ABTS 77.40 inhibition (%).

These results show that the SNFE had an expressive antioxidant activity in both methods tested. They were performed in a simple reaction environment, as described above. These results will not necessarily be the same in a biologically complex medium. To investigate the antioxidative capacity of the SNFE in complex environment, the burst respiratory method was used and the results are shown in Fig. 2. This graph shows the activation of human neutrophils by PMA in the absence (neutrophils control) or in the presence of neutrophils added to SNFE. The data were analyzed by the ANOVA test and complemented by the Turkey test. For neutrophils control, the average (n = 14) was 32,497.35 ± 4469.41 cintillations per minute (CPM), for the mass of 20 μg of SNFE was 13,408.35 ± 1939.23 CPM and 30 μg of SNFE was 11,403.28 ± 2177.58 CPM and 40 μg of SNFE was 9716.57 ± 1658.43 CPM.

The respiratory burst showed that the higher mass had better antioxidant activity (9716.57 CPM) compared to the control (32,497.35 CPM), similar to the ABTS and DPPH methods, indicating that even in a complex reaction medium as the cellular environment, the extract was able to maintain high antioxidant capacity.

As discussed previously, other species of *Nectandra* present the flavonoids as a class of secondary metabolites. On that basis, flavonoids contents characterization and quantification in SNFE was evaluated by photoacoustic spectroscopy (PAS) and flavonoid test, respectively.

The optical absorption spectrum by PAS of SNFE is shown in Fig. 3(A) and the result of the Gaussian deconvolution used to characterize the main wavelengths for the spectrum can be seen in Fig. 3(B).

Gaussian deconvolution of SNFE (Fig. 3(B)), suggests that the maximum absorption peak at 234 nm and 390 nm are the bands II (ring A) and I (ring B) of flavonoids, respectively. This suggests the presence of flavonoids in the extract, as seen in other species (Ribeiro et al., 2002).

The content of flavonoids in SNFE was determined and showed the value of 41.72 ± 1.92 μQE/mg extract, confirming the presence of this class of compounds with known antioxidant activity.

The goal of this work was to develop a novel product for treatment and/or prevention of skin damage. To accomplish this, the permeability across skin needed to be evaluated. Therefore, the SNFE was incorporated in a base topical formulation previously described, containing 1% of SNFE. The final formulation and the base (without SNFE) were analyzed by PAS in order to confirm their absorption profiles.

Results are presented in Fig. 3(A and B). They show that SNFE had a wide range of absorption, from ultraviolet to visible region (200–800 nm). Fig. 3(B) presents the centers absorbers obtained by Gaussian deconvolution. They suggest the possible species responsible for such absorption. The topical formulation with 1% of SNFE presents bands in positions similar to the SNFE spectrum, but with

Table 5
Adjusted models for phenolic contents and antioxidant activity of *Nectantra falcifolia* extracts produced according a central composite 2^2 design.

Dependent variables	Models	R^2	*p* value	Equation
Total phenolic content (PC)	$\widehat{PC} = (648.19 \pm 7.12) + (253.45 \pm 10.7) \cdot ETL - (150.00 \pm 11.25) \cdot ETQ - (268.59 \pm 10.07) \cdot PPL + (114.91 \pm 11.25) \cdot PPQ$	0.79	<0.05	(3)
Antioxidant activity (AA)	$\widehat{AA} = (23.58 \pm 0.20) - 6.26 \pm 0.28) \cdot ETL + (1.96 \pm 0.32) \cdot ETQ + (4.31 \pm 0.28) \cdot PPL + (1.70 \pm 0.32) \cdot PPQ$	0.78	<0.05	(4)

ETL: extraction time linear; ETQ: extraction time quadratic; PPL: plant proportion linear; PPQ: plant proportion quadratic.

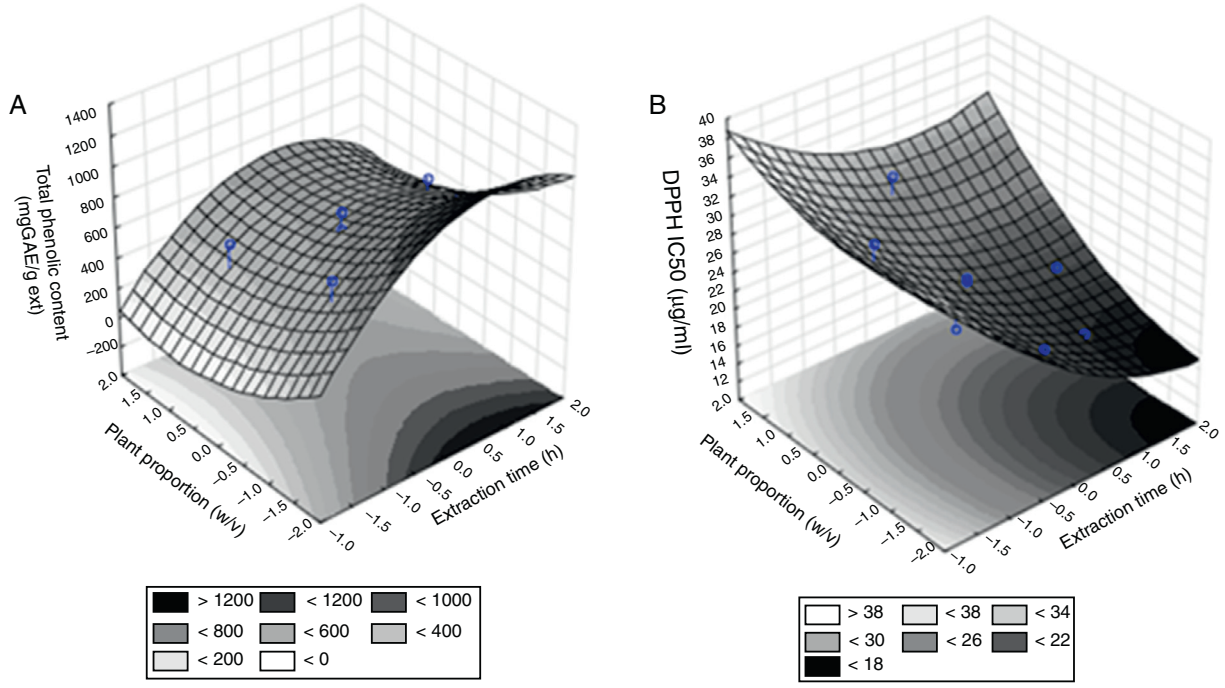

Fig. 1. Response surface plot from central composite 2^2 for the total phenolic content (A) and for antioxidant activity (B) in *Nectandra falcifolia* extracts

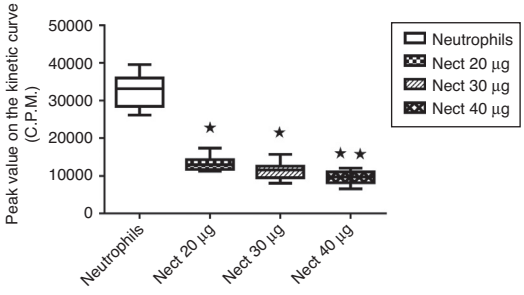

Fig. 2. Graph–Box plot of the peak values on the kinetic curves. Data were analyzed by ANOVA complemented by Turkey's $^{*}p < 0.001$ compared to control neutrophils; $^{**}p < 0.001$ compared to control neutrophils and 0.01 compared to *Nectandra* 20 µg. C.P.M.: count per minute.

a lower intensity. Base formulation spectrum is also presented in Fig. 3(A); it did not show absorption.

For evaluating skin penetration, the frequency of modulation used and the thermal diffusivity of the skin was $d = 4 \times 10^{-4}$ cm^2/s. The thermal diffusion length, in which the photoacoustic signal generated, was approximately 23 µm. Considering that samples had an average thickness of 770 microns, thicker than the penetration capacity of the signal, the samples were excited at the epidermal and dermal side and evaluated.

To illustrate, in Fig. 4(A) and (B) are presented the results of the PAS spectrum of skin samples of one individual of G3 and G4, respectively. Fig. 4 (A) shows data for the lower dose (2 mg/cm^2). It indicates the presence of the emulsion with SNFE in the epidermis, as evidenced by increases in optical absorption observed in treated skin compared to non treated (290 and 320 nm, 450 nm and 470 and 650 and 665 nm). The 650 and 665 nm optical absorption assigned to the chlorophylls presented in SNFE (Cunha et al.,

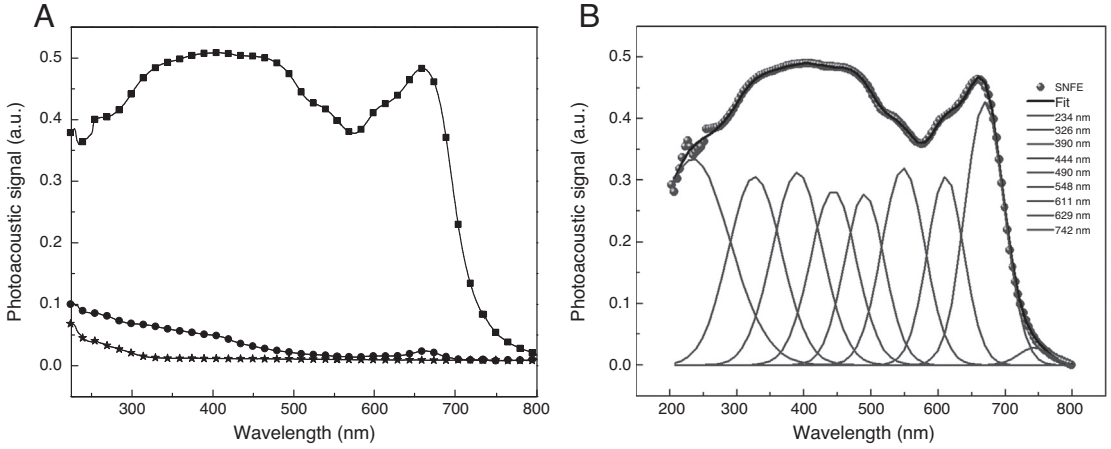

Fig. 3. Spectrum by PAS of (A) the standardized *N. falcifolia* extract (SNFE) – superior line, the emulsion without extract – inferior line and the emulsion with 1% of SNFE – intermediate; and (B) SNFE spectrum and the result of Gaussian deconvolution presenting the main absorption wavelengths (234, 326, 390, 444, 490, 548, 611, 629 and 742 nm).

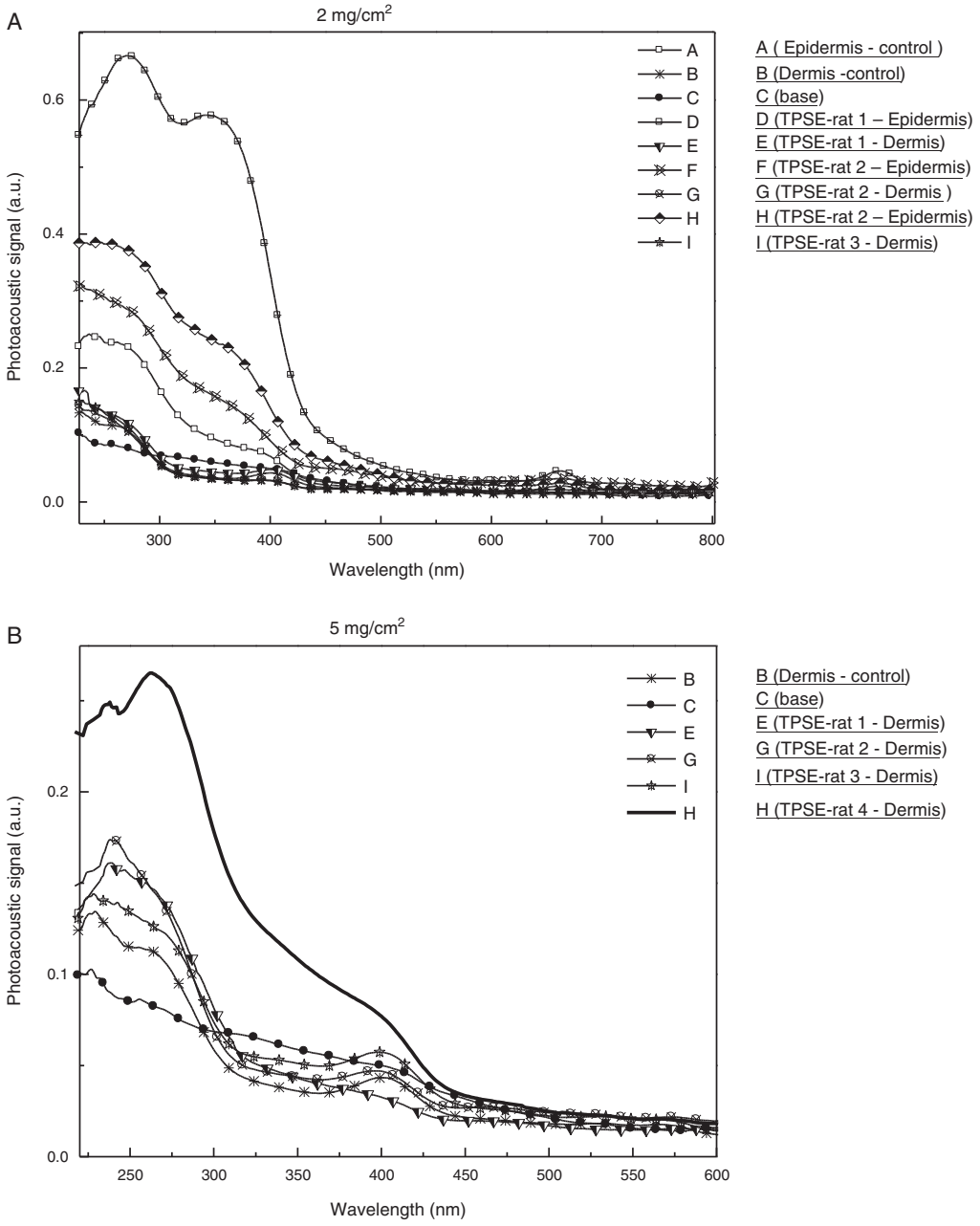

Fig. 4. Optical absorption spectrum obtained by PAS of TPSA in the epidermal and dermal side of the skin samples without application of products (epidermis control and dermis control) and faces the epidermal and dermal samples of skin with TPSA doses (A) 2 mg/cm^2 and (B) 5 mg/cm^2.

2009). Regarding the dermis, the present data indicates that little or no permeation occurred, since the only notable difference is a slight increase in the optical absorption near 250 nm region.

Fig. 4(B) (higher dose −5 mg/cm^2) shows the presence of the topical formulation containing SNFE in the dermis and epidermis, as indicated by the increased optical absorption in all region between 240 and 410 nm.

The components of base formulation did not contribute to the increased absorption between 240 and 300 nm, since it was not observed in the dermis. These results show that the film thickness of the product applied to the skin is another factor to be considered in terms of the product penetration.

Considering the capacity of SNFE across skin and anti-leishmanial, antimicrobial and antiinflammatory activities previously stated (Oliveira de Melo et al., 2006; Truiti et al., 2005, 2006), can be postulated new studies for tegumentar leishmania or bacterial infections on skin, as well inflammatory process that used to be mediated by oxidative process.

In summary, the data shows that the statistical experimental design could correctly guide the standardization of extract of *N. falcifolia*, when high antioxidant activity is desired. This extract could cross the skin barrier and is therefore a potential novel product for skin diseases and protection. However, more experiments are necessary to confirm this probable profile in humans and in toxicity studies.

Authors' contributions

FBBP, LMT contributed in running the laboratory work and analysis. FBBP, MCTT, AD contributed in the drafting of the data and in the writing of the manuscript. MCTT and AD supervised the laboratory work and contributed to critical reading of the manuscript. FY

contributed to the experimental design analysis. ACN and MLB contributed to the photoacoustic spectroscopy analysis. LH contributed to the permeation analysis. KLB and DSB contributed to the complementary antioxidative activity assays. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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